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Effect of blockade of nitric oxide in heart tissue levels of Renin Angiotensin System components in acute experimental Chagas disease

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ABSTRACT

Chagas disease (CD) is an important cause of cardiomyopathy in South America. The pathophysiology of CD is still a matter of debate. Renin Angiotensin System (RAS) components are clearly involved in cardiovascular diseases. RAS molecules interact with nitric oxide (NO) pathway in blood vessel and heart tissue. Thus, the aim of this study is to investigate possible changes in RAS molecules during the infection with Y strain *T. cruzi* and in response to acute administration of an inhibitor of the enzyme NO synthase, L-NAME. Male Holtzman rats were inoculated intraperitoneally with Y strain *T. cruzi* and received L-NAME or tap water from one day before the infection until 13 or 17 days post infection (dpi). Angiotensin converting enzyme 1 (ACE1) levels were significantly higher at day 17 when compared to baseline in atrium, whereas, in ventricle, ACE2 levels were significantly higher in 13 dpi when compared to baseline. In response to L-NAME treatment, atrium tissue levels of ACE1 were significantly reduced in treated animals at day 17, while Angiotensin-(1–7) concentration in atrium significantly increased in this group at the same time-point. No changes were detected in RAS components in the ventricle. ACE2 levels in Soleus muscle were significantly reduced in treated animals at day 13. In conclusion, changes in RAS molecules were detected during acute phase of *T. cruzi* infection and the inhibition of NO synthesis clearly interfered with expression of ACE1 and Angiotensin-(1–7) in the atrium.

1. Introduction

Chagas Disease (CD) is a zoonosis caused by the protozoan *Trypanosoma cruzi*, occurring from the southern of United States to Patagonia, Argentina [1,2]. Due to its high prevalence, morbidity and mortality, CD is a serious medical and social problem in South America, Central America and Mexico [1,2]. This disease is responsible for damage to various systems, mainly cardiovascular and digestive systems [2,3]. CD has been traditionally classified in two stages in humans: the acute phase, lasting from 10 to 60 days; and the chronic phase, without a defined duration, but starting just after the acute phase [2,3].

It is well known that some of components of the Renin Angiotensin System (RAS), as the heptapeptide Angiotensin-(1–7) [Ang-(1–7)] [4], Angiotensin-(1–9) [Ang-(1–9)] [5] and the more recently identified molecule Alamandine [6], exert cardio and renoprotective effects, which include reduction of oxidative stress, inflammation and fibrosis in heart and kidney tissues [7]. These peptides are formed through the hydrolysis of Angiotensin I (Ang I) or Angiotensin II (Ang II) by several enzymes as, for example, angiotensin converting enzyme 2 (ACE2) [8,9], a zinc metalloprotease, homologous to angiotensin converting enzyme (ACE), which converts Ang II directly into Ang-(1–7) or generates Ang-(1–9) from Ang I hydrolysis [10]. Ang-(1–9) can also be cleaved by ACE resulting in Ang-(1–7). However, Ang-(1–7) is mostly produced through the action of ACE2 on Ang II, which has 400-fold more affinity to ACE2 [10,11]. Among these RAS molecules, Ang-(1–7) is the best characterized in regard to beneficial effects in kidney and

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heart tissues [7,12]. The actions of Ang-(1–7) are mediated by Mas receptor [7,13,14]. Ang-(1–7) produces vasodilation, reduces cell proliferation, tissue inflammation and fibrosis [12]. On the other hand, other RAS components, mostly Ang II, when excessively produced, can cause injury to several organs and systems by acting via angiotensin type 1 (AT_1) receptors [15]. Ang II is able to produce vascular damage by stimulating the recruitment of immune system cells and the proliferation of vascular smooth muscle cells [7]. Some studies support a role for Ang II in chronic phases of CD, mainly in Chagas cardiopathy [3]. It is possible that the production of Ang-(1–7) may serve as physiological mechanism to counteract the deleterious effects of Ang II during chronic phases of CD.

Many studies have also shown complex interactions between RAS molecules and nitric oxide (NO) [16–19]. One of the actions elicited by the binding of Ang-(1–7) to Mas receptor is the enzymatic induction of Nitric Oxide Synthase (NOS), which, in turn, increases the local production of NO [17,19]. Conversely, Ang II promotes vasoconstriction and endothelial dysfunction by, at least in part, the inhibition of NO release and signal transduction [20,21].

In the present study, a reversible NOS inhibitor, the compound N ($_\omega$)-nitro-L-arginine methyl ester (L-NAME), was used to investigate the interaction of NO with RAS molecules in experimental CD. Interestingly, a growing body of studies has shown paradoxical effects of L-NAME in relation to NO biology [22–24]. Chronic administration of L-NAME resulted in increased synthesis of NO, as an escape phenomenon [23]. For this reason, in the present study we investigated the acute effect of L-NAME administration in a well-established experimental model of CD induced by intraperitoneal inoculation of Y strain *T. cruzi* (300,000 trypomastigotes/50 g body weight) [25]. Thus, the aim of this study is to investigate possible changes in RAS molecules during the induction of experimental CD and in response to acute administration of L-NAME in the same experimental model.

2. Material and methods

2.1. Animals care and ethical issues

Male Holtzman rats, aged 27–29 days, were used in all experimental procedures. The animals were maintained in filter-topped cages at 21 °C, under conditions of 12-hour light/12-hour dark cycle. Care and anesthesia obeyed the guidelines for Laboratory Animals established by The National Institute of Health (Bethesda, MD, USA), as recommended by the Institute of Biological Sciences at the Federal University of Minas Gerais, Belo Horizonte, Brazil. The Ethical Review Board of our institution approved study protocol and all experimental procedures (CETEA/UFMG, Permit Protocol Number 340/2014).

2.2. Study protocol

2.2.1. Evaluation of RAS components during Chagas disease induction

Rats were inoculated intraperitoneally with Y strain *T. cruzi* (300,000 trypomastigotes/50 g body weight). Parasitemia was estimated by Brener's method [26] in 5 μL of blood obtained from the tail from day 3–17 post inoculation. Animals were killed before disease induction (baseline) and during the acute phase at days 13 and 17, under a mixture of ketamine and xylazine anesthesia. After sacrifice, heart and soleus muscle were carefully removed and stocked at −70 °C for posterior tissue analysis of RAS components. The days 13 and 17 post-infection were chosen since they correspond to the acute phase of the disease and would provide a kinetic analysis of the molecules evaluated.

2.3. Effect of L-NAME in RAS components during disease induction

L-NAME (Sigma Chemical Co) treatment (treated group) or tap water (non-treated group) started one day before *T. cruzi* inoculation and continued until the day before the sacrifice. L-NAME was administered in drinking water (40 mg/kg/day) by gavage. Both groups, L-NAME treated and non-treated, were killed during the acute phase of CD at days 13 and 17, under a mixture of ketamine and xylazine anesthesia. After death, heart and soleus muscle were carefully removed and stocked at −70 °C for posterior tissue analysis of RAS components.

2.4. Histolopathological and histoquantitative methods

Hearts were fixed in 4% phosphate-buffered paraformaldehyde for 24 h and routinely processed for Paraplast (Oxford Labware) embedding. At least 4 sections of each organ (7-μm-thick) stained with hematoxylin and eosin were analyzed at 70-μm intervals.

2.5. Measurement of RAS components

Fragments of heart tissue (atrium and ventricle) and soleus muscle were homogenized in an extraction solution (100 mg of tissue per milliliter), containing 0.4 M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM phenyl methyl sulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KIU aprotinin, using Ultra-Turrax. Lysates were centrifuged at 13,000*g* for 10 min at 4 °C, and supernatants were collected.

Samples were then thawed and tissue levels of ACE1, Ang II, ACE2 and Ang-(1–7) and ACE2 were measured by ELISA, according to the procedures supplied by the manufacturer (MyBioSource, San Diego, CA, USA). All kits applied sandwich ELISA technique, except for ACE measurement whose kit applied competitive ELISA method. Sensitivity of the assays was 1.0 pg/mL for ACE and ACE2; 3.9 pg/mL for Ang I; 2.0 pg/mL for Ang-(1–7); and 18.75 pg/mL for Ang II. The biochemical assessments were performed blind regarding the experimental protocols.

2.6. Statistical analysis

The software GraphPad Prism release 5.0 (GraphPad softaware, San Diego, CA, USA) was used for statistical analysis. Gaussian distribution was checked by Kolmogorov Smirnov test. Results were expressed as means and standard deviation or medians and interquartile range, when appropriate. Comparisons between two groups were made by Mann–Whitney or unpaired Student's *t*-tests, when appropriate. For Gaussian variables, comparisons between three groups were made by analysis of variance followed by Student Newman-Keuls test. For non-parametric data, comparisons between three groups were made by Kruskal-Wallis test followed by Dunn test. All statistical tests were two-tailed with significance level of $p < 0.05$.

3. Results

3.1. Heart tissue changes and parasitemia in experimental Chagas disease

One day before infection, both non-treated and L-NAME treated animals did not show any histopathological change (Fig. 1 - panels A and B). Thirteen days post *T. cruzi* inoculation (dpi), the hearts from non-treated *T. cruzi* infected rats exhibited sparse focal inflammatory infiltrates composed of mononuclear cells and occasional amastigote nests in both ventricles (Fig. 1 - panel C). At the same time-point, L-NAME treated infected animals had more intense myocarditis and tissue parasitism (Fig. 1, panel D). Inflammatory areas were detected throughout the myocardium and epicardium at 17 dpi (Fig. 1, panels E and F). L-NAME treated animals also had increased tissue inflammation and fibroblastic proliferation (Fig. 1 - panel F). Parasitemia was assessed in non-treated and L-NAME-treated *T. cruzi*-infected rats. Higher parasitemia was observed in L-NAME-treated group at day 13 post infection (Fig. 1G).

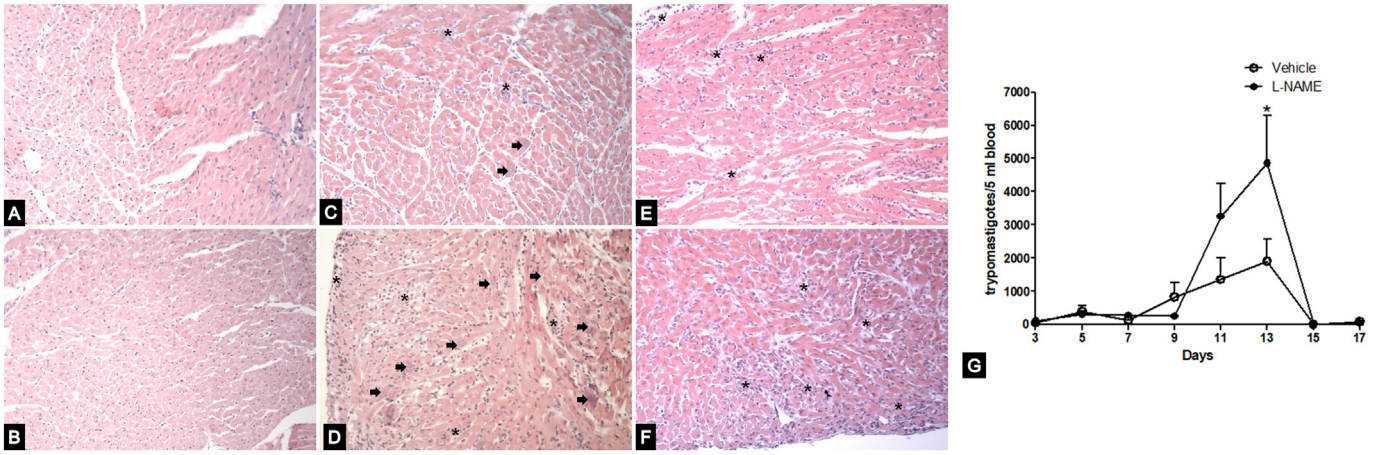

Fig. 1. Representative H&E stained sections of the heart from non-treated and L-NAME treated animals (n = 5 per group). Panels (A) and (B) represent heart tissue of rats at baseline, one day before *T. cruzi* inoculation. Panel (A) is from rats receiving tap water and panel (B) from animals receiving L-NAME for one day. Heart tissue from *T. cruzi* infected animals at 13 days post infection (dpi), non-treated with L-NAME, is represented in (C) and heart tissue from infected rats treated with L-NAME in (D). Heart tissue from non-treated infected animals at 17 dpi is represented in (E) and from infected animals treated with L-NAME in (F). Panels A and B show normal histological heart tissues. At 13 dpi: Focal infiltration of immune cells in myocardium (asterisks) and occasional amastigote nests (arrows) are present in non-treated infected animals (C). More intense myocarditis (asterisks) and tissue parasitism (arrows) are observed in infected rats treated with L-NAME (D). At 17 dpi: Multifocal inflammatory infiltration (asterisks) is observed in the myocardium of infected animals non-treated with L-NAME (E). Multifocal to coalescing and intense myocarditis and fibroblast proliferation (asterisks) are detected in infected rats treated with L-NAME (F). Original magnification: A–F: ×200. L-NAME treated *T. cruzi* infected rats presented increased parasitemia 13 days post infection, compared with non-treated *T. cruzi* infected animals (G).

3.2. Local RAS components in experimental Chagas disease

3.2.1. Atrium

Local RAS components were measured in the atrium at baseline, 13 and 17 days after the induction of CD (dpi).

Regarding the components of the classical RAS axis, following the infection, ACE1 levels were significantly higher in 17 dpi when compared to baseline [31.9 (25.4–42.1) versus 50.6 (49.2–55.3), p = 0.0081, Fig. 2 - panel A]. No differences were detected between baseline and 13 dpi and between 13 and 17 dpi (p > 0.05, Table 1). As also shown in Table 1, Ang II concentration in atrium did not change at baseline, 13 and 17 dpi (p > 0.05 for all comparisons).

Concerning components of the alternative RAS, ACE2 and Ang-(1–7) levels did not differ at baseline, 13 and 17 days after experimental induction of CD (p > 0.05 for all comparisons, Table 1).

3.2.2. Ventricle

Local RAS components were also measured in the ventricle at baseline, 13 and 17 days after the induction of CD (dpi).

ACE1 and Ang II levels in ventricle did not differ at baseline, 13 and 17 days after experimental induction of CD (p > 0.05 for all comparisons, Table 1).

ACE2 levels were significantly higher in 13 dpi when compared to baseline [24.8 (23.3–26.5) versus 28.4 (27.7–31-1), p = 0.015, Fig. 2 - panel B]. No differences were detected between baseline and 17 dpi, and between 13 and 17 dpi (p > 0.05, Table 1). Ang (1–7) levels did not change at baseline, 13 and 17 dpi (p > 0.05 for all comparisons), as also shown in Table 1.

3.2.3. Soleus muscle

In order to investigate the effects of CD on local RAS components of a noncardiac muscle tissue, experiments were made in the skeleton muscle soleus. RAS components were measured in soleus muscle tissue at baseline, 13 and 17 days after the induction of CD (dpi).

ACE1 and Ang II levels in soleus muscle tissue did not differ at baseline, 13 and 17 days after experimental induction of CD (p > 0.05 for all comparisons, Table 2).

ACE2 and Ang-(17) levels in Soleus muscle tissue did not differ at baseline, 13 and 17 days after experimental induction of CD (p > 0.05 for all comparisons, Table 2).

3.3. Modulation of local RAS components by L-NAME in experimental Chagas disease

3.3.1. Atrium

Local RAS components were also compared in heart tissue of the atrium at 13 and 17 days after the induction of CD in non-treated animals (13 dpi and 17 dpi) and in animals treated with L-NAME (13dpi + L-NAME and 17dpi + L-NAME).

ACE1 levels were significantly reduced in animals at 17 dpi treated with L-NAME in comparison to those at 17 dpi not receiving L-NAME (41.07 ± 1.66 versus 51.72 ± 1.86, p = 0.0081, Fig. 3 - panel A). No differences were detected in the comparison of rats with and without L-NAME at 13 dpi (p > 0.05, Table 3). Regarding Ang II concentrations, no changes were observed in response to L-NAME administration at 13 and 17 dpi (p > 0.05 for all comparisons, Tables 3 and 4).

Similar to Ang II, ACE2 levels did not differ in the comparison of the use versus non-use of L-NAME at both time points (p > 0.05 for all comparisons, Tables 3 and 4). On the other hand, levels of Ang-(1–7) in the atrium significantly increased at day 17 in rats receiving L-NAME when compared to untreated animals (118.8 ± 6.4 versus 97.3 ± 3.9, p = 0.03, Fig. 3 – panel B).

3.3.2. Ventricle

In the ventricle, local RAS components were compared in animals treated with L-NAME versus non-treated at 13 and 17 days after the induction of CD. There were no significant differences in all components of the both RAS axes at any time-point (p > 0.05 for all comparisons, Tables 3 and 4).

3.3.3. Soleus muscle

In soleus muscle tissue, local RAS components were compared in animals treated with L-NAME versus non-treated at 13 and 17 days after the induction of CD.

Similar to ventricle findings, ACE1 and Ang II levels did not differ in animals treated and nontreated with L-NAME at both time points (p > 0.05 for all comparisons, Table 5).

ACE2 levels were significantly reduced in animals at 13 dpi treated

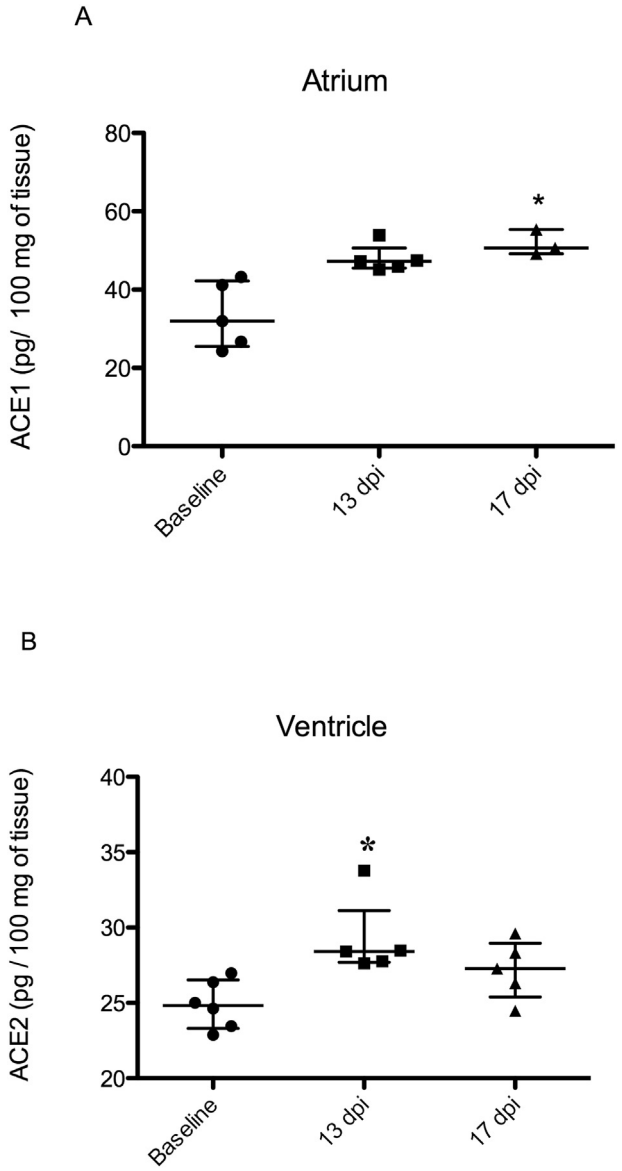

Fig. 2. A - Angiotensin converting enzyme (ACE1) concentrations (pg/100 mg of tissue) in heart tissue of the atrium at baseline, 13 (13 dpi) and 17 (17 dpi) days after experimental induction of Chagas disease. *p < 0.05 (Kruskal-Wallis test followed by Dunn post-test). B - Angiotensin converting enzyme 2 (ACE2) concentrations (pg/100 mg of tissue) in heart tissue of the ventricle at baseline, 13 (13 dpi) and 17 (17 dpi) days after experimental induction of Chagas disease. Data are expressed as individual values and bars represent median and interquartile range (n = 5 per group). *p < 0.05 (Kruskal-Wallis test followed by Dunn post-test).

Table 2
Concentrations of Renin Angiotensin System (RAS) components (pg/100 mg of tissue) in Soleus muscle at baseline, 13 and 17 days after experimental induction of Chagas disease. Data are expressed as mean ± standard deviation or median and interquartile range by brackets. p value was obtained by analysis of variance or Kruskal-Wallis test.

RAS components	At baseline	13 days	17 days	p value
ACE1	44.2 (38.4–58.4)	46.6 (40.9–52.6)	41.3 (25.4–42.6)	0.26
Ang II	158.3 ± 20.1	144.9 ± 17.7	147.9 ± 16.6	0.46
ACE2	29.6 (26.9–60.6)	38.2 (35.8–39.4)	29.5 (27.3–31.2)	0.11
Ang-(1–7)	112.0 ± 19.6	92.1 ± 16.2	109.4 ± 15.7	0.17

with L-NAME in comparison to those not receiving L-NAME (37.7 ± 1.1 versus 30.0 ± 2.3 p = 0.0151, Fig. 3 - panel C). No differences were detected in the comparison of rats treated and non-treated with L-NAME at 17 dpi (p > 0.05, Table 5). Regarding Ang-(1–7) concentrations, no changes were observed in response to L-NAME administration at 13 and 17 dpi (p > 0.05 for all comparisons, Table 5).

4. Discussion

The *T. cruzi* infection in Holtzman rats reproduces tissue parasitism and inflammation of the acute human disease [27,28]. In the present study, histopathological analysis of heart tissue showed increased of parasitemia and myocarditis in infected animals treated with L-NAME. Previous studies have shown that the administration of L-NAME reduced endogenous NO synthesis and, as a consequence, the resistance of mice to acute CD infection [26]. The more intense cardiac parasitism of L-NAME treated rats is in line with the concept of NO involvement in the control of experimental infection with *T. cruzi*. Indeed, NO and reactive oxygen species (ROS) release are crucial defense mechanisms against *T. cruzi* infection by promoting the killing of phagocytosed parasites by activated macrophages [29,30]. Moreover, cytokines production in response to infection may induce iNOS expression and NO release in macrophages [26,31,32]. However, excessive NO production seems to induce a detrimental oxidative stress response and has been often implicated in CD-associated heart diseases [26,31,32].

The discovery that RAS components are locally expressed in determined tissues, including the heart, pointed out to the role for this system in the pathogenesis of cardiac dysfunctions [12]. Accordingly, we found expression of all components in the atrium and ventricle of rats at baseline. After infection, there was a significant increase of the classical axis component, ACE1, in the atrium of infected mice at 17 dpi. Interestingly, a growing body of evidence has shown beneficial effects of ACE1 inhibitors, including captopril and enalapril, in acute CD-associated heart failure [33–36]. In line with our findings, A/J mice infected with Brazil strain of *T. cruzi* developed acute (21 days after infection) myocarditis, mainly characterized by severe focal inflammation, necrosis and fibrosis, which were reversed by captopril administration (5 mg/L in the water). Of note, captopril had no effect on parasitemia or cardiac parasite load [36]. Similar findings were also

Table 1
Concentrations of Renin Angiotensin System (RAS) components (pg/100 mg of heart tissue) in atrium and ventricle at baseline, 13 and 17 days after experimental induction of Chagas disease. Data are expressed as mean ± standard deviation and p value was obtained by analysis of variance.

Tissue	RAS components	At baseline	13 days	17 days	p value
Atrium	Ang II	171.4 ± 14.4	174.2 ± 22.1	166.4 ± 22.6	0.82
	ACE2	27.4 ± 4.1	28.4 ± 3.9	30.9 ± 2.1	0.30
	Ang-(1–7)	109.0 ± 9.4	119.2 ± 18.7	97.3 ± 7.8	0.09
Ventricle	ACE1	34.9 ± 4.1	30.6 ± 5.3	34.3 ± 8.9	0.49
	Ang II	100.1 ± 15.1	106.9 ± 29.3	113.8 ± 15.3	0.56
	Ang-(1–7)	59.2 ± 11.4	74.6 ± 20.4	74.1 ± 7.8	0.15

Legend: Ang II = Angiotensin II; ACE2 = angiotensin converting enzyme 2; Ang-(1–7) = Angiotensin-(1–7); ACE1 = angiotensin converting enzyme 1.

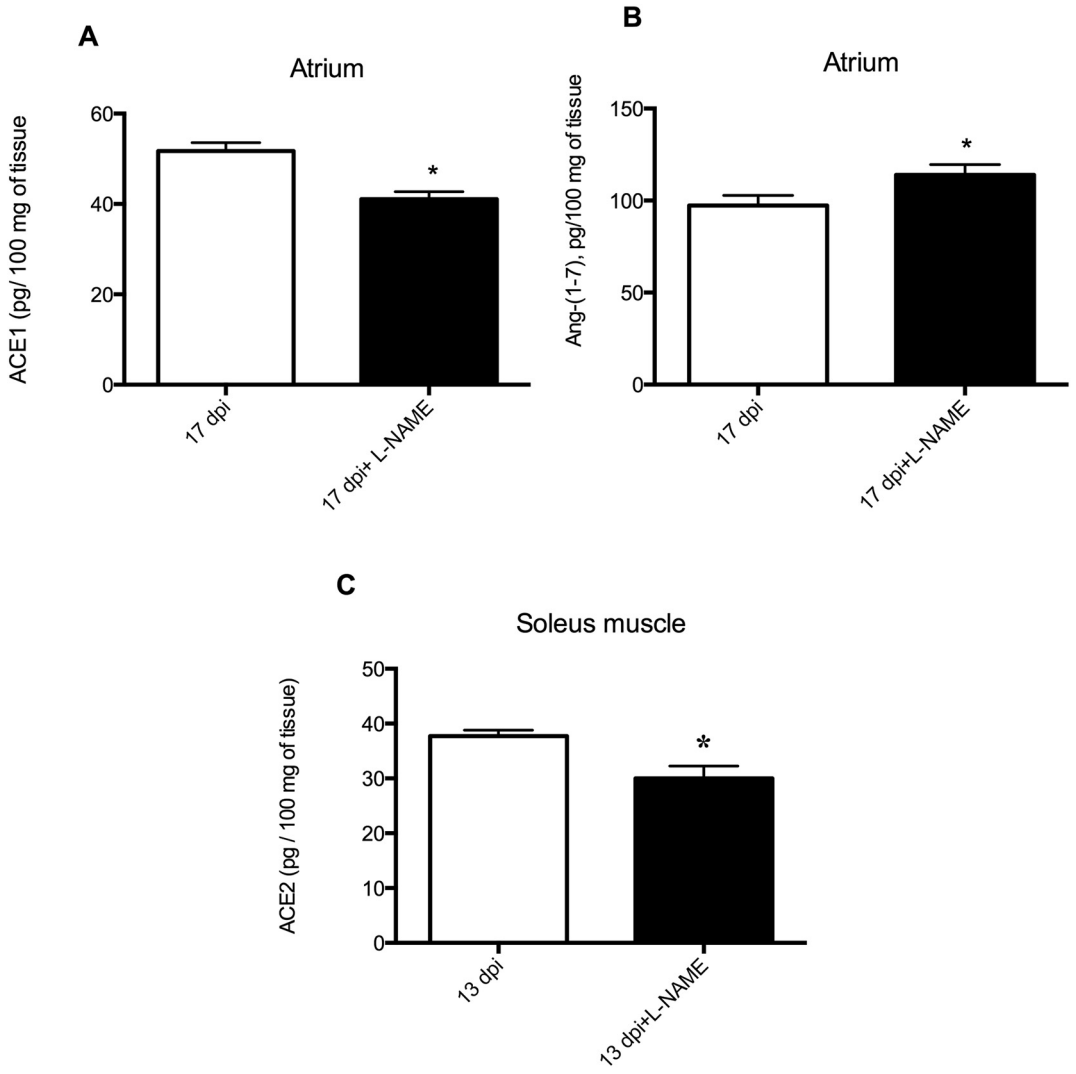

Fig. 3. A - Comparison of angiotensin converting 1 (ACE1) levels in heart tissue of the atrium in rats non-treated (17 dpi) and treated with L-NAME (17 dpi + L-NAME) at 17 days after induction of Chagas' disease. Bars represent means and standard deviation. *p < 0.05 (unpaired *t*-test). B - Comparison of Angiotensin-(1–7) [Ang(1–7)] levels in heart tissue of the atrium in rats non-treated (17 dpi) and treated with L-NAME (17 dpi + L-NAME) at 17 days after induction of Chagas' disease. Bars represent means and standard deviation. *p < 0.05 (unpaired *t*-test). C - Comparison of angiotensin converting 2 (ACE2) levels in soleus muscle of rats non-treated (13 dpi) and treated with L-NAME (13 dpi + L-NAME) at 13 days after induction of Chagas' disease. Bars represent means and standard deviation (n = 5 per group). *p < 0.05 (unpaired *t*-test).

Table 3
Concentrations of Renin Angiotensin System (RAS) components (pg/100 mg of heart tissue) in atrium and ventricle at 13 days after experimental induction of Chagas disease in animals non-treated (13 days) and treated with L-NAME (13 days + L-NAME). Data are expressed as mean ± standard deviation or median and interquartile range by brackets. p value was obtained by unpaired Student *t*-test or Mann-Whitney test.

Heart tissue	RAS components	13 days	13 days + L-NAME	p value
Atrium	ACE1	47.2 (45.5–50.6)	44.1 (38.8–54.5)	0.90
	Ang II	174.2 ± 9.9	181.2 ± 5.1	0.54
	ACE2	28.4 ± 1.7	30.9 ± 1.0	0.25
	Ang-(1–7)	119.2 ± 8.3	124.8 ± 5.1	0.58
Ventricle	ACE1	30.6 ± 2.3	31.2 ± 2.1	0.84
	Ang II	106.9 ± 13.1	120.5 ± 12.1	0.48
	ACE2	28.4 (27.7–31.1)	27.6 (25.5–28.2)	0.17
	Ang-(1–7)	64.2 (58.9–95-5)	68.2 (62.0–92.2)	0.69

Legend: Ang II = Angiotensin II; ACE2 = angiotensin converting enzyme 2; Ang-(1–7) = Angiotensin-(1–7); ACE1 = angiotensin converting enzyme 1.

Table 4
Concentrations of Renin Angiotensin System (RAS) components (pg/100 mg of heart tissue) in atrium and ventricle at 17 days after experimental induction of Chagas disease in animals non-treated (17 days) and treated with L-NAME (17 days + L-NAME). Data are expressed as mean ± standard deviation and p value was obtained by unpaired Student *t*-test.

Heart tissue	RAS components	17 days	17 days + L-NAME	p value
Atrium	Ang II	166.4 ± 10.1	158.2 ± 9.4	0.58
	ACE2	30.9 ± 0.9	28.2 ± 1.7	0.11
Ventricle	ACE1	34.3 ± 3.9	39.4 ± 1.5	0.28
	Ang II	113.8 ± 6.9	124.6 ± 14.1	0.51
	ACE2	27.2 ± 0.9	28.8 ± 1.1	0.27
	Ang-(1–7)	74.1 ± 3.5	68.4 ± 8.9	0.57

Legend: Ang II = Angiotensin II; ACE2 = angiotensin converting enzyme 2; Ang-(1–7) = Angiotensin-(1–7); ACE1 = angiotensin converting enzyme 1.

found in a study with 30 day-treatment with enalapril (25 mg/kg) that determined decrease in circulating levels of inflammatory mediators and NO along with a reduction in heart mononuclear cells infiltration in C57BL/6 mice infected with *T. cruzi* (Colombian strain) [33]. Anti-

Table 5
Concentrations of Renin Angiotensin System (RAS) components (pg/100 mg of tissue) in Soleus muscle at 13 and 17 days after experimental induction of Chagas disease in animals non-treated (13 days and 17 days) and treated with L-NAME (13 days + L-NAME and 17 days + L-NAME). Data are expressed as mean ± standard deviation or median and interquartile range by brackets. p value was obtained by unpaired Student *t*-test or Mann-Whitney test.

RAS components	13 days	13 days + L-NAME	p value
ACE1	30.6 ± 2.3	31.2 ± 2.1	0.84
Ang II	106.9 ± 13.1	120.5 ± 12.1	0.48
Ang-(1–7)	92.1 ± 16.2	109.4 ± 15.7	0.12

RAS components	17 days	17 days + L-NAME	p value
ACE1	41.3 (25.4–42.6)	33.2 (30.0–51.1)	0.55
Ang II	147.9 ± 7.4	161.4 ± 6.3	0.20
ACE2	29.5 (27.3–31.2)	30.9 (27.9–64.8)	0.69
Ang-(1–7)	97.4 ± 8.4	105.8 ± 4.8	0.41

Legend: Ang II = Angiotensin II; ACE2 = angiotensin converting enzyme 2; Ang-(1–7) = Angiotensin-(1–7); ACE1 = angiotensin converting enzyme 1.

inflammatory and cardioprotective effects of enalapril were also reported in mice after infection with the VL-10 strain of the *T. cruzi* [33]. Taken together, these studies support the role of ACE1 in heart dysfunction following acute CD, mainly associated with inflammatory process, as supported by our histopathological findings.

It has also been reported that the RAS counter-regulatory axis is involved in heart dysfunction [37,38]. In the current study, we found a significant increase in the levels of ACE2 in the ventricle of *T. cruzi* infected rats at 13 dpi. Interestingly, higher mRNA expression of ACE2 was found in the ventricular myocardium of subjects with idiopathic dilated cardiomyopathy or ischemic cardiomyopathy compared with donors with non-diseased hearts [37]. The concept that ACE2 is highly expressed in the falling heart corroborated the hypothesis that ACE2 may act to balance the activity of classical RAS components as an attempt to protect the cardiac tissue [37,39]. Specifically, in CD, increased activity of ACE2 was found in patients with heart failure, but not in patients without systolic dysfunction. Moreover, enhanced circulating levels of ACE2 were significantly correlated with clinical severity, worsening ventricular systolic dysfunction and other echocardiographic measures. More importantly, in a 3-year follow-up, high ACE2 activity was a predictive marker of mortality and heart transplant in CD patients [38].

There is a complex interaction between the components of the RAS and NO in the heart in the context of several cardiovascular diseases [40–43]. The current study is the first to provide evidence regarding these mechanisms in CD. Herein, the treatment with L-NAME significantly decreased ACE1 levels in the atrium of infected rats at 17 dpi compared with infected animals not receiving L-NAME. The administration of L-NAME is a well-established model of hypertension in rodents, leading to significant changes in cardiac function [41,43–46]. In contrast to the current findings, rats with hypertension induced by L-NAME exhibit increased ACE activity, among other changes, including oxidative stress imbalance, declined myocardial performance due to myocardial hypertrophy and fibrosis [43]. On the other hand, the administration of ACE inhibitors exerted a cardioprotective effect that last even in the absence of continued antihypertensive treatment [41,44–46]. Our contradicting result may indicate that the modulation of the RAS classical arm components by L-NAME occur in a context dependent manner. Further studies are necessary to better address this issue in response to infectious conditions, including CD.

The administration of L-NAME also influenced the RAS counter-regulatory axis, since an increase in Ang-(1–7) levels was found in the atrium of rats treated with L-NAME at 17 dpi if compared to non-treated infected animals. Interestingly, no changes in ACE2 levels were observed. It should be pointed out that other enzymes capable of directly or indirectly forming Ang-(1–7) are also present in the heart, such as prolyloligopeptidase [47] and cathepsin A [48]. There is evidence that Ang-(1–7) and its receptor Mas are expressed in the sinoatrial node cells of rats, which were associated with an anti-arrhythmogenic response [49]. A more recent study supported the protective role of Ang-(1–7) in cardiac arrhythmias, which were abrogated by the administration of L-NAME, indicating that Ang-(1–7) beneficial effects are dependent of NO [50]. Accordingly, acute exposure of cardiomyocytes to Ang-(1–7) induced NO release [51,52]. Herein, the lack of cardioprotection, as revealed by more intense inflammatory process in the heart, despite of the local increase of Ang-(1–7) might be, at least in part, explained by the inhibition of NO production by L-NAME.

In order to also investigate whether L-NAME can affect RAS components in response to CD in non-cardiac muscle tissue, we analyzed the skeletal muscle soleus. The treatment of L-NAME was able to decrease local levels of ACE2 in the soleus of infected animals at 13 dpi. It has been reported that *T. cruzi* infection promotes skeletal muscle damage by inducing mitochondrial dysfunction, muscle parasitism, cell necrosis, inflammation, and redox imbalance [53,54]. Importantly, NO seems to be crucial to increase skeletal muscle protein synthesis and to induce skeletal muscle regrowth, beneficial effects that are hampered by L-NAME administration [55]. Moreover, there is evidence supporting a protective role of ACE2 in skeletal muscles. For instance, in a murine model of Duchenne muscular dystrophy, high ACE2 activity in the skeletal muscle was associated with a reduction of local fibrosis and improvement of muscle function [56]. Accordingly, mice with genetic deletion of ACE2 had less muscle hypertrophy and impaired performance in voluntary running [57]. In this context, it is reasonable to hypothesize that the expression of ACE2 as well as its protective role in the skeletal muscle in CD might be also, at least in part, dependent of NO production.

In summary, our study showed, for the first time, the local expression of components of both RAS axes in atrium, ventricle and soleus muscle in an experimental model of CD. More importantly, we provided evidence for a critical role of NO in controlling T cruzi infection and in mediating cardioprotective effects of Ang-(1–7). Further studies are necessary to reveal the mechanisms beyond the complex interactions of RAS components and NO in CD.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

All the data used to support the findings of this study are available from the corresponding author upon request.

References

[1] A.B.B. de Oliveira, et al., Parasite-vector interaction of chagas disease: a mini-review, Am. J. Trop. Med. Hyg. 98 (3) (2018) 653–655.
[2] J.A. Pérez-Molina, I. Molina, Chagas disease, Lancet 391 (10115) (2018) 82–94.
[3] E.A. Bocchi, et al., Chronic Chagas heart disease management: from etiology to cardiomyopathy treatment, J. Am. Coll. Cardiol. 70 (12) (2017) 1510–1524.
[4] A.S. Santos Robson, J. Ferreira Anderson, C. Simões e Silva Ana, Recent advances in the angiotensin-converting enzyme 2–angiotensin(1–7)–Mas axis, Exp. Physiol. 93

(5) (2008) 519–527.
[5] M. Flores-Munoz, et al., Angiotensin-(1–9) attenuates cardiac fibrosis in the stroke-prone spontaneously hypertensive rat via the angiotensin type 2 receptor, Hypertension 59 (2) (2012) 300.
[6] R.Q. Lautner, et al., Discovery and characterization of alamandine. A novel component of the renin–angiotensin system, Circ. Res. 112 (8) (2013) 1104.
[7] E.S.A.C. Simoes, M.M. Teixeira, ACE inhibition, ACE2 and angiotensin-(1–7) axis in kidney and cardiac inflammation and fibrosis, Pharmacol. Res. 107 (2016) 154–162.
[8] M. Donoghue, et al., A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9, Circ. Res. 87 (5) (2000) E1–E9.
[9] S.R. Tipnis, et al., A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase, J. Biol. Chem. 275 (43) (2000) 33238–33243.
[10] C. Vickers, et al., Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase, J. Biol. Chem. 277 (17) (2002) 14838–14843.
[11] G.I. Rice, et al., Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism, Biochem. J. 383 (Pt 1) (2004) 45–51.
[12] T.R. Prestes, et al., The anti-inflammatory potential of ACE2/angiotensin-(1–7)/Mas receptor axis: evidence from basic and clinical research, Curr. Drug Targets 18 (11) (2017) 1301–1313.
[13] S.V.B. Pinheiro, et al., Genetic deletion of the angiotensin-(1–7) receptor Mas leads to glomerular hyperfiltration and microalbuminuria, Kidney Int. 75 (11) (2009) 1184–1193.
[14] R.A. Santos, et al., Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas, Proc. Natl. Acad. Sci. U. S. A. 100 (14) (2003) 8258–8263.
[15] E.S.A.C. Simoes, J.T. Flynn, The renin-angiotensin-aldosterone system in 2011: role in hypertension and chronic kidney disease, Pediatr. Nephrol. 27 (10) (2012) 1835–1845.
[16] A. Abwainy, et al., Endogenous angiotensin-(1–7)/Mas receptor/NO pathway mediates the cardioprotective effects of pacing postconditioning, Am. J. Physiol. Heart Circ. Physiol. 310 (1) (2016) H104–H112.
[17] A. Sobrino, et al., Mas receptor is involved in the estrogen-receptor induced nitric oxide-dependent vasorelaxation, Biochem. Pharmacol. 129 (2017) 67–72.
[18] Z.-T. Wu, et al., The PI3K signaling-mediated nitric oxide contributes to cardiovascular effects of angiotensin-(1–7) in the nucleus tractus solitarii of rats, Nitric Oxide 52 (2016) 56–65.
[19] G. Yang, et al., Angiotensin-(1–7)-induced Mas receptor activation attenuates atherosclerosis through a nitric oxide-dependent mechanism in apolipoproteinE-KO mice, Pflugers Arch. 470 (4) (2018) 661–667.
[20] L.A. Villalobos, et al., The angiotensin-(1–7)/Mas axis counteracts angiotensin II-dependent and -independent pro-inflammatory signaling in human vascular smooth muscle cells, Front. Pharmacol. 7 (482) (2016).
[21] X. Zhang, et al., Cellular basis of angiotensin-(1–7)-induced augmentation of left ventricular functional performance in heart failure, Int. J. Cardiol. 236 (2017) 405–412.
[22] I.M. Grumbach, et al., A negative feedback mechanism involving nitric oxide and nuclear factor kappa-B modulates endothelial nitric oxide synthase transcription, J. Mol. Cell. Cardiol. 39 (4) (2005) 595–603.
[23] J. Kopincova, A. Puzserova, I. Bernatova, L-NAME in the cardiovascular system - nitric oxide synthase activator? Pharmacol. Rep. 64 (3) (2012) 511–520.
[24] J. Kopincová, A. Púzserová, I. Bernátová, Biochemical aspects of nitric oxide synthase feedback regulation by nitric oxide, Interdiscip. Toxicol. 4 (2) (2011) 63–68.
[25] N.K. Oliveira, et al., Cardiac autonomic denervation and expression of neurotrophins (NGF and BDNF) and their receptors during experimental Chagas disease, Growth Factors 35 (4–5) (2017) 161–170.
[26] P. Petray, et al., Release of nitric oxide during the experimental infection with Trypanosoma cruzi, Parasite Immunol. 16 (4) (1994) 193–199.
[27] E.R. Camargos, et al., Infection with different Trypanosoma cruzi populations in rats: myocarditis, cardiac sympathetic denervation, and involvement of digestive organs, Am. J. Trop. Med. Hyg. 62 (5) (2000) 604–612.
[28] R.C. Melo, C.R. Machado, Trypanosoma cruzi: peripheral blood monocytes and heart macrophages in the resistance to acute experimental infection in rats, Exp. Parasitol. 97 (1) (2001) 15–23.
[29] S. Gupta, et al., Serum-mediated activation of macrophages reflects TcVac2 vaccine efficacy against Chagas disease, Infect. Immun. 82 (4) (2014) 1382–1389.
[30] S.-j. Koo, et al., Macrophages promote oxidative metabolism to drive nitric oxide generation in response to Trypanosoma cruzi, Infect. Immun. 84 (12) (2016) 3527–3541.
[31] M.M. Rodrigues, M. Ribeirao, S.B. Boscardin, CD4 Th1 but not Th2 clones efficiently activate macrophages to eliminate Trypanosoma cruzi through a nitric oxide dependent mechanism, Immunol. Lett. 73 (1) (2000) 43–50.
[32] J.S. Silva, F.S. Machado, G.A. Martins, The role of nitric oxide in the pathogenesis of Chagas disease, Front. Biosci. 8 (2003) s314–s325.
[33] G. de Paula Costa, et al., Enalapril prevents cardiac immune-mediated damage and exerts anti-Trypanosoma cruzi activity during acute phase of experimental Chagas disease, Parasite Immunol. 32 (3) (2010) 202–208.
[34] A.M. Khoury, et al., Acute effects of digitalis and enalapril on the neurohormonal profile of chagasic patients with severe congestive heart failure, Int. J. Cardiol. 57 (1) (1996) 21–29.
[35] A.L.J. Leite, et al., The immunomodulatory effects of the Enalapril in combination with Benznidazole during acute and chronic phases of the experimental infection with Trypanosoma cruzi, Acta Trop. 174 (2017) 136–145.
[36] J.S. Leon, K. Wang, D.M. Engman, Captopril ameliorates myocarditis in acute experimental Chagas disease, Circulation 107 (17) (2003) 2264–2269.
[37] A.B. Goulter, et al., ACE2 gene expression is up-regulated in the human failing heart, BMC Med. 2 (2004) 19.
[38] Y. Wang, et al., Plasma ACE2 activity is an independent prognostic marker in Chagas' disease and equally potent as BNP, J. Card. Fail. 16 (2) (2010) 157–163.
[39] M.A. Crackower, et al., Angiotensin-converting enzyme 2 is an essential regulator of heart function, Nature 417 (6891) (2002) 822–828.
[40] A. Abareshi, et al., Effect of angiotensin-converting enzyme inhibitor on cardiac fibrosis and oxidative stress status in lipopolysaccharide-induced inflammation model in rats, Int. J. Prev. Med. 8 (2017) 69.
[41] L.A. Biwer, et al., Protection against L-NAME-induced reduction in cardiac output persists even after cessation of angiotensin-converting enzyme inhibitor treatment, Acta Physiol (Oxf.) 207 (1) (2013) 156–165.
[42] L. Ibarra-Lara, et al., Fenofibrate therapy restores antioxidant protection and improves myocardial insulin resistance in a rat model of metabolic syndrome and myocardial ischemia: the role of angiotensin II, Molecules 22 (1) (2016).
[43] T. Silambarasan, et al., Sinapic acid prevents hypertension and cardiovascular remodeling in pharmacological model of nitric oxide inhibited rats, PLoS One 9 (12) (2014) e115682.
[44] T.M. Hale, et al., Short-term ACE inhibition confers long-term protection against target organ damage, Hypertens. Res. 35 (6) (2012) 604–610.
[45] M.M. Khattab, A. Mostafa, O. Al-Shabanah, Effects of captopril on cardiac and renal damage, and metabolic alterations in the nitric oxide-deficient hypertensive rat, Kidney Blood Press. Res. 28 (4) (2005) 243–250.
[46] F. Simko, et al., Continuous light and L-NAME-induced left ventricular remodelling: different protection with melatonin and captopril, J. Hypertens. 28 (Suppl. 1) (2010) S13–S18.
[47] M.A. Cicilini, et al., Heart prolyl endopeptidase activity in one-kidney, one clip hypertensive rats, Braz. J. Med. Biol. Res. 27 (12) (1994) 2821–2830.
[48] H.L. Jackman, et al., Angiotensin 1–9 and 1–7 release in human heart: role of cathepsin A, Hypertension 39 (5) (2002) 976–981.
[49] A.J. Ferreira, et al., The angiotensin-(1–7)/Mas receptor axis is expressed in sinoatrial node cells of rats, J. Histochem. Cytochem. 59 (8) (2011) 761–768.
[50] J.V. Joviano-Santos, et al., New insights into the elucidation of angiotensin-(1–7) in vivo antiarrhythmic effects and its related cellular mechanisms, Exp. Physiol. 101 (12) (2016) 1506–1516.
[51] M.A. Costa, et al., Angiotensin-(1–7) upregulates cardiac nitric oxide synthase in spontaneously hypertensive rats, Am. J. Physiol. Heart Circ. Physiol. 299 (4) (2010) H1205–H1211.
[52] M.F. Dias-Peixoto, et al., Molecular mechanisms involved in the angiotensin-(1–7)/Mas signaling pathway in cardiomyocytes, Hypertension 52 (3) (2008) 542–548.
[53] A.L. Baez, et al., Mitochondrial dysfunction in skeletal muscle during experimental Chagas disease, Exp. Mol. Pathol. 98 (3) (2015) 467–475.
[54] R.D. Novaes, et al., Parasite control and skeletal myositis in Trypanosoma cruzi-infected and exercised rats, Acta Trop. 170 (2017) 8–15.
[55] A.F. Aguiar, et al., Nitric oxide synthase inhibition impairs muscle regrowth following immobilization, Nitric Oxide 69 (2017) 22–27.
[56] C. Riquelme, et al., ACE2 is augmented in dystrophic skeletal muscle and plays a role in decreasing associated fibrosis, PLoS One 9 (4) (2014) e93449.
[57] D. Motta-Santos, et al., Effects of ACE2 deficiency on physical performance and physiological adaptations of cardiac and skeletal muscle to exercise, Hypertens. Res. 39 (7) (2016) 506–512.